Remarks

Upon entry of the foregoing amendment, claims 136-174 are pending in the application, with claims 136 and 142 being the independent claims. Claims 138, 143, 157-161, and 163-170 are withdrawn. Claims 136, 137, 139-142, 144-156, 162, and 171-173 are sought to be amended. Claim 174 is sought to be added. Support for the amendments to claims 136 and 142 and for new claim 174 may be found, *e.g.*, at paragraph [154] of the specification. The claims have also been amended to recite "abortive promoter cassette" in place of "molecule." In addition, claim 151 has been amended to recite RNA as a target molecule. Support for these amendments may be found throughout the specification. The specification has been amended to indicate that a benefit application has issued as a U.S. patent.

Based on the above amendments and the following remarks, Applicant respectfully requests that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Rejections under 35 U.S.C. § 112, second paragraph

The Examiner rejected claims 136, 137, 139-142, 144-156, 162 and 171-173 as allegedly indefinite. Applicant respectfully traverses these rejections.

Specifically, the Examiner rejected claim 136 and claims dependent thereon allegedly because a proper Markush group has not been identified. Applicant has amended the claim to recite "and" between the second and third members of the group.

The Examiner also rejected claims 139-141 because it is allegedly unclear what is meant by "further comprising a promoter." Applicant has amended the claim to specify that the promoter is a transcription promoter.

The Examiner rejected claim 142 and claims dependent thereon because the claim allegedly fails to mention elements (a) and (b) and further because elements (c) and (g) are allegedly duplicated. Applicant has deleted element (g) and has renamed the elements so that they start with (a).

The Examiner rejected claim 149 and 150 because there is allegedly insufficient antecedent basis for "said nucleic acid." Applicant has amended the claim to recite "said oligonucleotide."

Based on the foregoing, Applicant respectfully requests that the Examiner reconsider and withdraw the rejections.

Rejection under 35 U.S.C. § 102

The Examiner has rejected claims 136, 137, 139-142, 144-146, 151-155, 171 and 172 under 35 U.S.C. § 102(b) as allegedly anticipated by Munroe *et al.* (U.S. Patent No. 5,597,694.[1] The Examiner also rejected claims 136, 137, 139-142, 144-147, 151-156 and 171 under 35 U.S.C. § 102(b) as allegedly anticipated by Daube *et al.* (*PNAS*, 91:9539-9543 (1994))("Daube I") as evidenced by Daube *et al.* (*Science* 258:1320-1324)("Daube II"). Applicant respectfully traverses this rejection.

Under 35 U.S.C. § 102, a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior

[1]Applicants note that, on page 7 of the Office Action, the Examiner indicates that "[c]laims ????" are rejected. However, the Examiner indicates in subsequent paragraphs which claims are rejected.

art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987).

Solely to advance prosecution, and not in acquiescence of the Examiner's rejection, Applicant has amended claims 136 and 142 to recite that when the target specific linker is a nucleic acid, the linker comprises a single-stranded overhang region of 5 to 40 nucleotides. Neither Munroe *et al.* nor any of the Daube *et al.* documents teach an abortive promoter cassette that comprises a single stranded overhang of 5 to 40 nucleotides.

Munroe *et al.* describe that the nucleic acid fragments are ligated to an oligonucleotide by blunt-end ligation. *See* col. 2, lines 45-60. Blunt-ended nucleic acids do *not* have any nucleotide overhang. Thus, it is clear that the oligonucleotide of Munroe *et al.* lack "a single-stranded overhang region of 5 to 40 nucleotides."

Daube I describe an oligonucleotide having a Bam HI restriction site. A Bam HI restriction digest leaves a 4 base-pair overhang. Daube II note the presence of two restrictions sites in the duplex, *viz.*, a Sma I site and a Sal I site. Applicant notes that digestion by Sma I leaves blunt ends, and therefore does not leave any nucleotide overhang. A Sal I restriction digest results in a 4 base-pair overhang. Thus, the oligonucleotides of Daube I and II lack "a single-stranded overhang region of 5 to 40 nucleotides." As discussed in more detail below, Applicant submits neither Munroe *et al.*, nor Daube I or Daube II, alone or in combination, provide any reason to make bubble oligonucleotides with overhangs of 5 to 40 nucleotides.

Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

Rejection under 35 U.S.C. § 103

The first 103 rejection

The Examiner rejected claims 136, 137, 139-142, 144-148, 151-156, and 171-173 as allegedly unpatentable over Daube I as evidenced by Daube II in view of Berninger *et al.* (U.S. Patent No. 5,194,370). The gist of the Examiner's rejection is that it would have been obvious to make a bubble complex (as allegedly taught by Daube I and Daube II) with a target specific linker having a single stranded overhang region from about 10 to about 25 nucleotides (as allegedly taught by Berninger *et al.*). Applicant respectfully traverses these rejections.

Applicant disagrees with the Examiner's analysis. Considering the cited art as a whole, there is no reason that would have prompted a person of ordinary skill in the art to create an abortive promoter cassette bubble complex of claim 136 part (a)(ii) having a linker that comprises a single-stranded overhang region of 5 to 40 nucleotides. Applicants respectfully submit that the Examiner's rejection, both the suggestion/motivation and reasonable expectation of success, is based on hindsight analysis in view of the Applicant's claimed invention. Daube I and II provide no suggestion, and in fact, teach away from any suggestion or motiviation to create an overhang of 5 to 40 nucleotides. First, both Daube I and II were solely interested in investigating the nucleic acid framework and *mechanisms* of functional transcription elongation complexes and termination. Daube II constructed a synthetic RNA-DNA duplex to mimic this framework. *Id.* at p. 1320-21. Daube I ligated a construct to the oligonucleotide to investigate termination mechanisms.

Daube I and II describe use of this complex *solely* as a tool to probe the properties of a functional elongation complex and termination mechanisms. Both Daube

I and II note the presence of restriction sites in the duplex. Daube I use the Bam HI site *solely* for purposes of making a synthetic construct, by ligating a nucleic acid construct to study mechanisms of termination. Applicants assert that persons skilled in the art of molecular biology and recombinant DNA technology are well aware that recombination of DNA is practically and efficiently achieved by use of type II restriction enzymes. This approach is by far the most common and economical and the approach that was taken by Daube I in ligating the nucleic acid construct to the complex. The type II restriction endonucleases usually recognize 4-6-base pair (bp) sites on DNA and cleave each site in a separate reaction. The enzymes that recognize 4-6 bp sites leave no more than a 4 bp overhang for subsequent ligation and cloning. Applicant notes that there are a few type II enzymes that exist that recognize 8 bp sites, however, Bilcock *et al.* (*Journal of Biological Chemistry*, 274:36379-36386 (1999))(**EXHIBIT A**) demonstrate the unsuitability of these enzymes for use as restriction enzymes. *See* Abstract.

Daube I use the restriction site *solely* for the purpose of ligating a double stranded nucleic acid construct to the complex, to create a synthetic construct that can both initiate and terminate transcription. Thus, there is no suggestion or motivation in Daube I to generate an overhang of 5-40 nucleotides in length in the bubble complex, because doing so would inevitably frustrate attaching, via ligation, a double stranded nucleic acid to the oligonucleotide. Ligating an nucleic acid fragment to an overhang of 5-40 nucleotides would require additional steps, such as a gap filling step with a polymerase, and subsequent purification and isolation of the DNA. A person skilled in the art would have no reason to undertake such additional steps to make a synthetic construct. The easiest and most efficient means is to digest with a type II restriction enzyme, as performed by Daube I, which leaves no more than a 4 bp overhang, and perform a ligation reaction.

Thus, for at least these reasons, Daube I teach away from any suggestion or motivation of performing the modification suggested by the Examiner.

Daube II appear to utilize the Sma I and Sal I restriction site to study processivity, by generating templates of different sizes. Thus, Daube II do not even contemplate use of any overhang generated by the restriction enzyme as a means to attach a nucleic acid. In addition, there is no reason to use a restriction enzyme generating an overhang of 5 to 40 nucleotides, for purposes of generating templates of different sizes as shown by Daube II, because, *e.g.*, Bilcock *et al.* teach that type II enzymes recognizing 8 bp sites are unsuitable for use as restriction enzymes, except in limited circumstances, none of which apply here.

Berninger *et al.* similarly does not provide a reason to modify the Daube I or Daube II constructs. Berninger *et al.* discloses reporter driven templates. Specifically, Berninger *et al.* describe a method referred to as "ligation activated transcription." The method allegedly produces nucleic acid sequence end products that are substantially identical to the starting products. Berninger *et al.* attach an RNA promoter to a single stranded DNA and make multiple RNA copies of the single stranded DNA. The RNA is then converted back into cDNA by extension of a primer by reverse transcriptase. The RNA template is removed by RNase H. Columns 3-6 of Berninger *et al.* indicate steps in the method. Berninger *et al.* indicate that "when all of these components are provided and the conditions are satisfied, target nucleic acid sequences that may be present in the reaction volume may be amplified to form product DNA and product RNA." There is no suggestion or motivation in Berninger *et al.* to substitute the proto-promoter with a bubble complex.

In addition, Daube I does not provide any reason to use the bubble complex to detect a target nucleic acid. In fact, Daube I, contrary to the Examiner's assertions, *teach away* from any expectation that transcription of a bubble complex and a promoter-driven template, as taught by Berninger *et al.*, are equivalent, and thus, there is no reason to substitute one for the other. Importantly, Daube I compared the termination efficiency of transcription from a promoter driven template and bubble complex and found that the termination efficiency of the bubble complex was very low (efficiency of 19%) compared with that of a promoter template (efficiency of 88%). *See* p. 9541 of Daube I. Daube I offered various possible theories for the low termination efficiency of bubble complex transcription and speculated that the lowered efficiency

> might reflect the tendency of the nascent RNA to rehybridize to the template DNA during *E.coli* RNA polymerase-catalyzed transcript elongation from the bubble duplex, since the resulting rehybridized transcript might be unable to form a termination hairpin (or other element of RNA secondary structure) required for intrinsic termination and RNA release.

Id. Daube I conducted experiments to further investigate the inefficiency of termination of transcripts generated from the bubble complex. The results of the experiments ostensibly show that an RNA trap is necessary to improve efficiency to prevent reannealing of the nascent RNA to the non-complementary bubble sequence. Daube I hypothesized that the "residual level of termination obtained in the absence of an RNA trap can be attributed to the low level of RNA displacement that does occur under these conditions." *Id.* at 9542. Daube I speculated about three possible mechanistic schemes, shown on p. 9542, to interpret the results. Daube I highlights the investigational nature of the complex by noting that "these complexes may also be useful in studying other regulatory aspects of transcript elongation and termination, such as pausing, attenuation,

factor mediated anti-termination, and rho-dependent termination." *Id.* at 9542. Daube I also emphasize the need for additional comparative studies of promoter driven and bubble duplex transcription:

> [f]urther comparative studies with promoter bubble-less duplex constructs may prove useful in separating such promoter dependent effects from effects reflecting only the process of elongation.

Id. at p. 9543. Based on such description and characterization by Daube I of the complex, including differences of the complex in transcription termination *vis-a-vis* promoter generated transcription termination, Applicant respectfully asserts that there is no reason to use the abortive promoter cassette complex of claim 136 part (a)(ii) for diagnostic purposes, let alone making a modification to the complex attaching a target specific linker having an overhang of 5 to 40 nucleotides. Moreover, due to the investigational nature of the complex as a mechanistic tool, as discussed extensively in both Daube I and Daube II, Applicants respectfully submit that the skilled artisan would have no reasonable expectation that ligating or attaching a target sequence to the complex would have worked in a diagnostic assay. Based on the disclosure of Daube I, Daube II, or Berninger *et al.*, either alone in combination, it is not known or suggested how the complex would function in a complex milieu of, *e.g.*, a biological or environmental sample for diagnostic analysis. Daube I and Daube II present a study of the complex under controlled, artificial conditions, and even then, note that there are differences of the complex compared to a promoter driven complex. The whole focus of Daube I is the investigational nature of this construct as a mechanistic tool to probe properties of transcription. For at least these reasons, the Examiner has not established a *prima facie* case of obviousness.

Accordingly, Applicant respectfully requests that the Examiner reconsider and withdraw the rejection.

The second 103 rejection

The Examiner also rejected claim 162 under 35 U.S.C. § 103(a) as allegedly unpatentable over Daube I as evidenced by Daube II in view of Berninger *et al.* and further in view of Kim *et al.* (U.S. Patent No. 5,846,723). The Examiner contends that while neither Daube I, II, or Berninger *et al.* explicitly disclose that the linker should be specific for telomerase, Kim *et al.* disclose a well known practice for detecting telomerase activities for the purposes of detecting malignant cancers. Applicant respectfully traverses this rejection.

As indicated above, considering the cited art as a whole, there is nothing found in Daube I, Daube II or Berninger *et al.*, either alone or in combination, that would prompt the artisan to create an abortive promoter cassette bubble complex of claim 136 part (a)(ii) having a linker that comprises a single-stranded overhang region of 5 to 40 nucleotides. Therefore, it is not material whether or not it was a well known practice for detecting telomerase activities for the purpose of detecting malignant cancers.

Accordingly, Applicant respectfully requests that the Examiner reconsider and withdraw the rejection.

Obviousness-type Double Patenting

The Examiner provisionally rejected claims 136, 137, 139-142, 144-148, 151-156, 162 and 171-173 for obviousness-type double patenting over various claims 23-31 and 35-43 of copending Application No. 10/976,240. The Examiner contends that the

claims of the '240 application are drawn to a narrower species of the generic construct as claimed in the instant application. The Examiner contends that while claims 23-31 of the '240 application recite a generic term "abortive promoter cassette," in view of the figures of the '240 application referencing an abortive promoter cassette, the constructs are identical to the construct defined in the instant application, and therefore, deemed obvious over each other.

Applicant respectfully requests that the Examiner hold the present rejection in abeyance, pending the identification of otherwise allowable subject matter, at which time Applicant will consider filing any necessary terminal disclaimers.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Exhibit A

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Reactions of Type II Restriction Endonucleases with 8-Base Pair Recognition Sites*

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Type II restriction endonucleases usually recognize 4–6-base pair (bp) sites on DNA and cleave each site in a separate reaction. A few type II endonucleases have 8-bp recognition sites, but these seem unsuited for restriction, since their sites are rare on most DNA. Moreover, only one endonuclease that recognizes a target containing 8 bp has been examined to date, and this enzyme, *Sfi*I, needs two copies of this site for its DNA cleavage reaction. In this study, several endonucleases with 8-bp sites were tested on plasmids that have either one or two copies of the relevant sequence to determine if they also need two sites. *Sgf*I, *Srf*I, *Fse*I, *Pac*I, *Pme*I, *Sse*8781I, and *Sda*I all acted through equal and independent reactions at each site. *Asc*I cleaved the DNA with one site at the same rate as that with two sites but acted processively on the latter. In contrast, *Sgr*AI showed a marked preference for the plasmid with two sites and cleaved both sites on this DNA in a concerted manner, like *Sfi*I. Endonucleases that require two copies of an 8-bp sequence may be widespread in nature, where, despite this seemingly inappropriate requirement, they may function in DNA restriction.

The recognition sequences for the majority of the ~3,000 type II restriction endonucleases identified to date are symmetrical palindromes of DNA, 4–6-bp[1]-long, although ~20 enzymes of this type recognize 8-bp targets (1). The latter are particularly valuable as tools for the analysis of genomic DNA because they usually cleave DNA into larger DNA fragments than the enzymes cutting at 4- or 6-bp sequences, due to the relative rarity of their sites (2). However, doubts exist over whether a restriction enzyme with an 8-bp site could provide a bacterial cell with an effective defense against foreign DNA (3). Restriction demands at least one recognition site on the incoming DNA, and its efficiency increases with the number of sites (4). The probability of DNA escaping restriction, by being modified at all sites before being cleaved at any one site, declines logarithmically with the number of sites. Yet an 8-bp sequence must occur less frequently on DNA than any 4- or 6-bp element from that sequence, so phage or plasmid DNA may often lack an 8-bp site or contain only a small number of such sites. Hence, if the defense against phage infections by a restriction-modification system confers a selective advantage to a bacterial cell, evolutionary pressures should result in a contraction of the length of the recognition sequence (5).

Most of the current information about the mode of action of type II restriction enzymes derives from a relatively small number of enzymes, almost all of which recognize either a 4- or a 6-bp sequence, such as *Bam*HI, *Eco*RV, *Mun*I, *Pvu*II, or *Taq*I (Refs. 6–10, and references therein). In each of these examples, the protein is a dimer of identical subunits that interacts symmetrically with a palindromic DNA sequence, so that the two active sites in the enzyme are positioned on the scissile phosphodiester bonds in each strand. In the presence of Mg^{2+}, the cofactor for DNA cleavage, the two strands are cut in parallel reactions. The cleavage of both strands is normally completed before the enzyme dissociates from the DNA, although, in some instances, the enzyme dissociates after cutting just one strand and then returns to that site to cut the second strand (11). On DNA with multiple sites, these enzymes usually act in a distributive manner at each individual copy of the recognition sequence. However, they sometimes act processively on a DNA with two or more sites. For example, *Eco*RI can cleave one site, translocate to another site by an intramolecular process, cut that site, and only then leave the DNA (12, 13). Conversely, the restriction enzymes in the type IIe group, such as *Eco*RII and *Nae*I, require two copies of their recognition sequence (14, 15). Both *Eco*RII and *Nae*I are reported to be homodimeric proteins that have two distinct DNA-binding sites. One binding site has the catalytic functions for DNA cleavage, but this remains inactive unless a second copy of the recognition sequence binds to an allosteric site elsewhere in the dimer (16–18). The DNA at the allosteric site is not cleaved (19, 20).

To date, a reaction mechanism has been established for only one of the type II enzymes that recognizes a site with 8 specified bp: the *Sfi*I endonuclease from *Streptomyces fimbriatus* (21–27). In contrast to both the orthodox enzymes such as *Eco*RV and the type IIe enzymes such as *Eco*RII, *Sfi*I is a tetrameric protein that has two identical binding sites for its palindromic recognition sequence, each presumably made from two subunits. However, *Sfi*I has no activity when only one DNA-binding site is occupied. Instead, it has to bind two copies of its recognition sequence before being able to cleave DNA. The two sites can be in *cis*, on the same molecule of DNA, or in *trans*, on separate molecules of DNA. In the former case, *Sfi*I tethers the intervening DNA in a loop, while, in the latter, it bridges the two DNA molecules. As with other proteins that span two sites (28), *Sfi*I prefers sites in *cis* over sites in *trans*. It generally cleaves plasmids with two sites more rapidly than plasmids with one site. Moreover, the turnover of *Sfi*I on plasmids with two sites is normally completed by the liberation of DNA cut in both strands at both sites; only a small fraction of

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[1] The abbreviations used are: bp, base pair; kb, kilobase pair(s); MCS, multiple cloning site; SC, supercoiled; OC, open circle; FFL, full-length linear; L1 and L2, linear DNA fragments from the cleavage of a circular DNA with two sites at both sites.

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the DNA is liberated after cutting just one site. The concerted action of *Sfi*I at two recognition sites is reminiscent of the enzymes that mediate DNA rearrangements by site-specific recombination or transposition (29), but a role for *Sfi*I in rearrangements has been rejected (30). Like the orthodox restriction enzymes (31), phosphodiester hydrolysis by *Sfi*I inverts the stereoconfiguration of the phosphate, so its reaction cannot involve a covalent enzyme-DNA intermediate (32). Enzymes that catalyze both DNA breakage and religation normally conserve the energy of the phosphodiester bond, a prerequisite for the religation step, by forming a covalent intermediate (29).

In vivo, *Sfi*I can restrict DNA that has two or more *Sfi*I sites, but it is incompetent at restricting DNA with one site (30). The mode of action of *Sfi*I thus exacerbates the doubts over whether an enzyme recognizing an 8-bp sequence could defend a cell against phage or plasmid DNA. However, the recognition sequence for *Sfi*I is unusual (33) in that it contains 8 specified bp but these are interrupted by a unspecified spacer of 5 bp (Table I). Apart from one isoschizomer of *Sfi*I, all of the other type II enzymes that cleave DNA at 8-bp sites recognize uninterrupted sequences of 8 consecutive bp (Table I). Hence, while the type II enzymes with continuous recognition sites of 8 bp might act like *Sfi*I, they may differ from *Sfi*I and behave instead like the orthodox enzymes such as *Bam*HI or *Eco*RV. These possibilities were examined by assaying several restriction enzymes on plasmids that have either one or two copies of the relevant sequence in order to determine whether they act concertedly at two sites or cleave each site in a separate reaction.

EXPERIMENTAL PROCEDURES

Enzymes—Restriction endonucleases were purchased from the following suppliers: *Asc*I, *Fse*I, *Pac*I, *Pme*I, *Sse*8387I, *Sac*I, *Sal*I, and *Sph*I from New England Biolabs; *Sgf*I from Promega; *Srf*I from Stratagene; and *Sda*I from Fermentas; *Sgr*AI from both Roche Molecular Biochemicals and New England Biolabs (with equivalent results). Enzyme concentrations are given in terms of units of enzyme activity, as specified by the supplier. Other enzymes were obtained from New England Biolabs or Roche Molecular Biochemicals.

DNA—The plasmids pAT153 (34) and pNEB193 (New England Biolabs) have been described before; the latter is identical to pUC19 (35) except for an enlarged multiple cloning site (MCS) with a number of additional restriction sites. Two derivatives of pAT153, pDB7 and pDB8 (Fig. 1*a*), and one of pNEB193, pAB1 (Fig. 1*b*), were constructed by standard methods (36). The duplex used in the construction of pDB7 was produced by annealing two 50-base synthetic oligodeoxynucleotides that were complementary to each other except for 4 bases at their 5′ termini; the resultant 46-bp duplex had 4-base 5′-extensions that matched an *Eag*I terminus at one end and a *Sty*I terminus at the other (Fig. 1*a*). The *Sgr*AI site in this duplex had the same sequence as the intrinsic site on pAT153, with respect to both its purine/pyrimidine degeneracies (Table I) and to its flanking sequence for 3 bp on either side. The duplex used in the construction of pDB8 was made by the same procedure; it had the same sequence as the duplex for pDB7, except that it lacked the *Sgr*AI site and its 4-base 5′-extensions matched, respectively, *Eco*RI and *Hin*dIII termini (Fig. 1*a*). The plasmids were used to transform *rec*A strains of *Escherichia coli*, either HB101 (36) or ER2238 (37). The transformants were cultured in M9 minimal medium with 1 mCi/liter [*methyl*-^{3}H]thymidine, and the covalently closed form of the plasmid was purified by density gradient centrifugations (38). The preparations were largely supercoiled monomeric plasmid, with <10% as either dimeric plasmid or nicked open circle DNA.

Assays—Reactions were carried out at 37 °C in 200-μl volumes and were initiated by adding the restriction enzyme (typically 10 units) to the requisite plasmid (10 or 20 nM) in an appropriate buffer. In the first instance, the buffer used with each enzyme was that advised by the supplier. To test for processivity (12), most enzymes were also examined in modified buffers with elevated ionic strengths. For enzymes where the recommended buffer contains NaCl, the modified buffer had double the concentration of NaCl. Similarly, buffers with KOAc were adjusted to twice the advised level of KOAc. At various times after adding the enzyme, aliquots (15 μl) were removed from the reactions and mixed immediately with 10 μl of an EDTA stop mix (38). The samples were analyzed by electrophoresis through agarose under conditions that separated the supercoiled substrate and each of the various products from the reaction (Fig. 2). The segments of the agarose gel that encompassed the substrate and each product were analyzed individually by scintillation counting to yield the concentration of each form of the DNA at each time point (38). For plasmids with two recognition sites, the two linear DNA fragments (L1 and L2) arising from cleavage at both sites were counted together to obtain a single value for the concentration of doubly cut DNA (L1/2).

Experimental Strategy—A distinction between the different modes of action seen among the type II restriction enzymes can be made by analyzing the reaction kinetics of the enzyme on two substrates, one with one copy of its recognition sequence and another with two copies.

An orthodox enzyme like *Eco*RV will cleave a circular DNA with one copy of its recognition site first in one strand, converting the supercoiled (SC) substrate to the nicked open circle (OC) form of the DNA, and then in the second strand to produce the full-length linear (FLL) form (Fig. 2*a*). However, the hydrolysis of both phosphodiester bonds is often much faster than the dissociation of the cleaved DNA (8, 13, 39). In these cases, the nicked form exists only as a transient enzyme-bound intermediate, and the sole product that accumulates during a steady-state reaction is the FLL form. A SC DNA with two sites will be cleaved by an orthodox enzyme acting distributively first at one site to yield FLL DNA and then at the other site to give two linear fragments, L1 and L2 (Fig. 2*b*). The reaction on a two-site DNA should therefore follow a sequential A → B → C pathway, where the concentration of B first rises and then falls in a manner specified by the relative rates of the A → B and the B → C steps and where the formation of C is preceded by a characteristic lag phase (40). If the recognition sites on the one- and two-site substrates are all equal to each other, then the initial rate for the utilization of the SC DNA with one site (v_1) should equal that for the utilization of the SC DNA with two sites (v_{2A}), and the latter should also equal the rate for the conversion of the FLL DNA to L1 and L2 (v_{2B}).

A type II restriction enzyme that acts processively on a DNA with multiple sites should also utilize the two-site substrate at the same rate as the one-site substrate. But if the enzyme then travels along the DNA to another site and cuts that site before departing from the DNA, the SC substrate with two sites will be converted quickly to the doubly cut products, without an intervening accumulation of FLL DNA and without a lag phase preceding the formation of L1 and L2. However, the processivity is unlikely to be 100% efficient, and the enzyme will sometimes depart from the DNA before cutting the second site. Nevertheless, processivity will diminish the yield of FLL DNA from the two-site substrate, relative to that from an orthodox enzyme acting distributively. Moreover, the degree of processivity is likely to decrease as the ionic strength of the reaction is increased (12, 13). Hence, an enzyme that acts processively at low ionic strength is likely to act in a distributive manner at high ionic strength.

In contrast, a restriction enzyme that follows the mechanism proposed for the type IIe enzymes will utilize the substrate with two sites more rapidly than that with one site, since the interaction with the second site, which is needed to activate the enzyme, will be aided if this is provided in *cis* rather than in *trans*. Having cleaved one site on a two-site substrate, a type IIe enzyme would cleave the residual site at a slow rate, so a large amount of FLL DNA should accumulate during the reaction.

An enzyme that acts concertedly at two recognition sites, like *Sfi*I, would also utilize a two-site plasmid more rapidly than a one-site plasmid. However, in contrast to a type IIe enzyme, a concerted reaction on a two-site plasmid will give directly the final products cut at both sites, L1 and L2. A diminished yield of FLL DNA from a SC DNA with two sites could thus be due to either concerted or processive actions, but these can be distinguished by analyzing the reactions at varied ionic strengths. Both the difference in the reaction rates of *Sfi*I on plasmids with one or two sites and the degree of concertedness on plasmids with two sites depend on the concentration of NaCl in the reaction (22). In reactions lacking NaCl, *Sfi*I cleaves DNA with one site almost as readily as DNA with two sites; under these conditions, the protein binds to its recognition sites with sufficiently high affinity so that even the relatively weak interactions with sites *in trans* still permit the maximal reaction rate (27). A small amount of salt prevents the interactions in *trans*, so *Sfi*I reactions on DNA with one site are blocked at lower levels of NaCl than those on DNA with two sites (22). In high salt, *Sfi*I cleaves DNA with two sites much more rapidly than DNA with one site. But the progressive destabilization of the complex of *Sfi*I and two DNA sites with increasing ionic strength results in the progressive liberation of products from the two-site substrate that have been cleaved in three,

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two, or one phosphodiester bonds in place of the product cleaved at both sites in both strands (25, 26).

The reaction kinetics of a restriction enzyme on one-site and two-site substrates thus provide a diagnostic test for the mode of action of the enzyme; for independent reactions at individual sites, $v_1 = v_{2A} = v_{2B}$; for processive action on a DNA with two sites, $v_1 = v_{2A} < v_{2B}$; for activation by a second site, $v_1 < v_{2A} > v_{2B}$; for concerted action at two sites, $v_1 < v_{2A} < v_{2B}$. However, data at one ionic strength that yield a match to one of the above sets of relative values for v_1, v_{2A}, and v_{2B} may be insufficient for the diagnosis. An unambiguous distinction between these possibilities requires data at varied ionic strengths.

A meaningful comparison of the activities on one- and two-site substrates requires the following conditions to be met. First, the plasmids must be isolated from recombination-deficient strains to prevent the one-site DNA from recombining to its dimeric form with two sites; *rec*A strains were used here. Second, the DNA sequences flanking each recognition site on the two substrates must all be the same, since restriction activity is often affected by the flanking DNA (41–43); plasmid substrates were designed to meet this requirement (Fig. 1). Third, the two-site substrate must have an appropriate length of DNA between the sites. Lengths of <300 bp may be inappropriate, because DNA looping between closely spaced sites depends on both the helical periodicity and the bending of the intervening DNA (26). Conversely, on linear DNA, the stability of a loop between sites separated by >300 bp decreases as the separation increases, but this effect is largely nullified by DNA supercoiling (28). On SC DNA, increasing the separation of the sites above 400 bp has at most only a marginal effect on loop stability (44, 45). All of the tests described here used SC plasmids with, in the case of the two-site substrates, >500 bp between the sites. Fourth, the reactions must employ lower concentrations of enzyme than substrate. Otherwise, the enzyme may bind independently to each site on the two-site DNA and cleave this DNA at double the rate of the one-site DNA.

This study used commercial preparations of restriction enzymes, whose concentrations were specified in terms of units of activity rather than molarity. Nevertheless, the reactions are likely to have used lower concentrations of enzyme than DNA. For several enzymes, varied numbers of units were added to the reactions, and in all cases, the reaction velocities increased linearly with the number of units (data not shown). This behavior is characteristic of a steady-state reaction with substrate in excess of the enzyme. If the reactions had contained enzyme in excess of substrate, the rates would not have varied with the amount of enzyme. A further concern arises from the use of SC substrates. If an enzyme is more active on SC DNA than on linear DNA (or *vice versa*), the rate for the conversion of a SC DNA with two sites to the FLL form will differ from that for the subsequent conversion of FLL DNA to L1 and L2. Several of the enzymes were therefore tested on both SC and linear DNA substrates, the latter being generated by cleaving the plasmid with another restriction enzyme; in all cases, the SC and linearized substrates gave the same reaction rates (data not shown).

TABLE I
Recognition sequences

The names, the species of origin, and the recognition sequences (1) for all of the restriction enzymes examined here are listed.

Enzyme	Species[a]	Recognition sequence[b]
*Sfi*I	*Streptomyces fimbriatas*	GGCCNNNNN ↓ NGGCC
*Sgf*I	*Streptomyces griseoruber*	GCGAT ↓ CGC
*Srf*I	*Streptomyces* sp.	GCCC ↓ GGGC
*Fse*I	*Frankia* sp.	GGCCGG ↓ CC
*Sgr*AI	*Streptomyces griseus*	GR ↓ CCGGYG
*Sse*8387I	*Streptomyces* sp.	CCTGCA ↓ GG
*Sda*I[c]	*Streptomyces diastalicus*	CCTGCA ↓ GG
*Asc*I	*Arthobacter* sp.	GG ↓ CGCGCC
*Pac*I	*Pseudomonas alcaligenes*	TTAAT ↓ TAA
*Pme*I	*Pseudomonas mendocina*	GTTT ↓ AAAC
*Sac*I	*Streptomyces achromogenes*	GAGCT ↓ C
*Sal*I	*Streptomyces albus* G	G ↓ TCGAC
*Sph*I	*Streptomyces phaeochromogenes*	GCATG ↓ C

[a] In some instances, the species within the genus has not been identified.
[b] ↓, the point of cleavage; N, any base; R, purine; Y, pyrimidine.
[c] *Sda*I is an isoschizomer of *Sse*8387I.

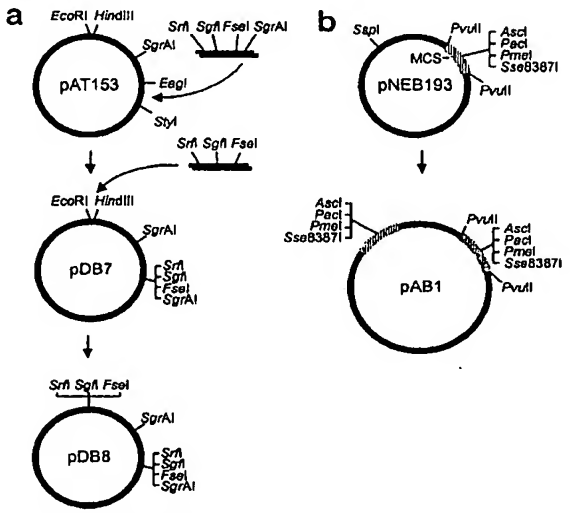

FIG. 1. **Construction of plasmid substrates.** *a*, the plasmid pAT153 was cleaved with *Eag*I and *Sty*I and ligated to an oligonucleotide duplex whose sequence included individual recognition sites for *Sgr*AI, *Sgf*I, *Srf*I, and *Fse*I. The resultant plasmid, pDB7, was cleaved with *Eco*RI and *Hin*dIII and ligated to an oligonucleotide duplex whose sequence included individual recognition sites for *Sgf*I, *Srf*I, and *Fse*I to yield pDB8. The vector, pAT153, has one recognition site for *Sgr*AI; pDB7 has two sites for *Sgr*AI and one each for *Sgf*I, *Srf*I, and *Fse*I; and pDB8 has two sites for *Sgf*I, *Srf*I, and *Fse*I. *b*, the plasmid pNEB193 was cleaved with *Pvu*II, and the fragment spanning the MCS, shown in *gray shading*, was isolated by electrophoresis and ligated to a second sample of pNEB193 that had been cleaved with *Ssp*I. The MCS in pNEB193 has solitary recognition sites for *Asc*I, *Pac*I, *Pme*I, and *Sse*8387I (*Sda*I) and also, not shown, for *Sac*I, *Sal*I, and *Sph*I, while pAB1 has two copies of each of these sites.

RESULTS

*Sgf*I, *Srf*I, *Sgr*AI, *and Fse*I—The above strategy was applied first to a set of four enzymes with 8-bp recognition sites: *Sgf*I, *Sgr*AI, *Srf*I, and *Fse*I. The first three of these were selected because they, like *Sfi*I, are from *Streptomyces* species (Table I). *Fse*I was chosen because its recognition sequence is the same as that for *Sfi*I except for the absence of the 5-bp interruption in the *Sfi*I site (Table I). Plasmids with one or two recognition sites for each enzyme were constructed from pAT153, which has one site for *Sgr*AI and none for the other enzymes (Fig. 1*a*). The first construct, pDB7, contains two sites for *Sgr*AI, separated by 571 bp, and one site for the other enzymes. The second, pDB8, contains two sites for *Sgf*I, *Srf*I, and *Fse*I, separated in each case by 949 bp.

For each enzyme, parallel experiments were carried out on the one-site and two-site plasmids. Samples were withdrawn from the reactions at varied times and analyzed by electrophoresis through agarose. Typical gels, from *Sgf*I reactions on pDB7 and pDB8, are shown in Fig. 2, *a* and *b*, respectively (others not shown). With both plasmids, the intact SC, the nicked OC, and the FLL forms of the DNA were isolated from each other, as were the fragments produced by cutting both sites on the two-site DNA, L1 and L2. The concentrations of each form of the DNA, at each time point sampled during the reaction, were then determined (Fig. 3). Rates for the utilization of the one- and two-site substrates, v_1 and v_{2A}, respectively, were measured from the initial linear decline in the concentration of SC DNA with time, while the rate for the second reaction on the two-site substrate, v_{2B}, was assessed relative to v_{2A} from the time course for the production and decay of the FLL DNA. If $v_{2A} = v_{2B}$, the maximal amount of FLL DNA produced during the reaction will be 40% of the total DNA, but if the amount of FLL DNA rises to a maximum of >40% of the total DNA, then $v_{2A} > v_{2B}$; conversely, a maximum of <40% indicates that $v_{2A} < v_{2B}$ (modeling not shown).

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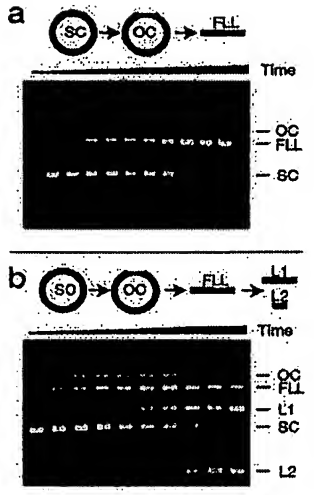

FIG. 2. **Reactions of a type II endonuclease on plasmids with one or two recognition sites.** The reactions contained 50 units·ml^{-1} *Sgf*I and 20 nM DNA (~95% supercoiled) in 10 mM Tris·HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, at 37 °C. The DNA in *a* was pDB7, which has one *Sgf*I site, and the DNA in *b* was pDB8, which has two *Sgf*I sites. At timed intervals after adding the enzyme, samples from the reactions were quenched with stop mix and analyzed by electrophoresis through agarose. The *schematics* in both *a* and *b* illustrate the various forms of DNA that can exist during these reactions. The agarose gels in both *a* and *b* illustrate the separation of these forms of the DNA; the electrophoretic mobility of each form is marked on the *right* of the gels. The reaction times (0–120 min) are noted as *expanding scales above* the *gels*.

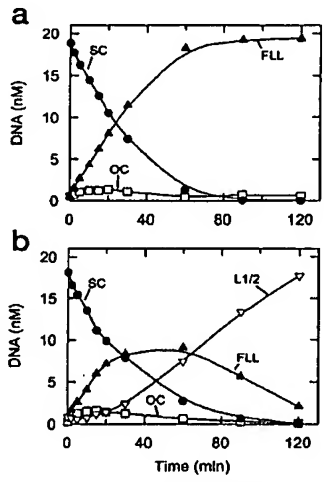

FIG. 3. ***Sgf*I on plasmids with one or two recognition sites.** The reactions of *Sgf*I on pDB7 (which has one *Sgf*I site) (*a*) or pDB8 (two *Sgf*I sites) (*b*) that are shown in Fig. 2, *a* and *b*, respectively, were analyzed to obtain the concentrations of the following forms of the DNA at each time point during the reaction: ●, SC; □, OC; ▲, FLL; ▽ (only in *b*), total DNA in the two final products cut at both sites (L1/2). The plasmids were ^{3}H-labeled, and the DNA concentrations were determined by assessing individual segments of the agarose gels (Fig. 2) in a scintillation counter.

The reaction of *Sgf*I on a SC plasmid with one cognate site yielded virtually none of the OC DNA. Instead, almost all of the substrate was converted directly to the FLL product (Fig. 3*a*). Thus, as with many other restriction enzymes (11), *Sgf*I cuts its recognition site in both strands before dissociating from the DNA. *Sgf*I again yielded virtually none of the OC DNA from the plasmid with two *Sgf*I sites, and the only product formed in significant yield during the initial period of this reaction was

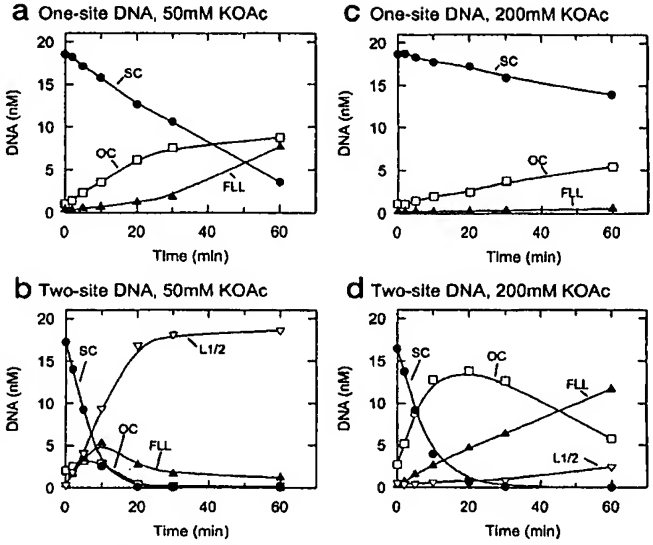

FIG. 4. ***Sgr*AI on plasmids with one or two recognition sites.** Reactions at 37 °C contained 50 units·ml^{-1} *Sgr*AI and 20 nM DNA (~90% supercoiled) in 33 mM Tris acetate (pH 7.9), 10 mM magnesium acetate, 0.5 mM dithiothreitol and either 50 mM KOAc (*left panels*; *a* and *b*) or 200 mM KOAc (*right panels*; *c* and *d*). In the *two upper panels* (*a* and *c*), the DNA was pAT153, which has one *Sgr*AI site. In the *two lower panels* (*b* and *d*), the DNA was pDB7, which has two *Sgr*AI sites. Samples taken from the reactions at timed intervals were analyzed as above to obtain the concentrations of the following forms of the DNA: SC (●), OC (□), FLL (▲), and total DNA in the two final products cut at both sites (L1/2) (▽; only in *b* and *d*).

FLL DNA; the FLL DNA was subsequently cleaved at the second site to give L1 and L2 (Fig. 3*b*). Both the initial rise in the concentration of FLL DNA, to a maximum of ~40% of the total DNA, and the lag phase preceding the formation of L1 and L2 denote a sequential A → B → C pathway, with equal rates for the A → B and the B → C steps. Moreover, the rate at which *Sgf*I utilized the one-site substrate (v_1 = 0.41 nM·min^{-1}) equaled that for the utilization of the two-site substrate (v_{2A} = 0.44 nM·min^{-1}). The *Sgf*I endonuclease thus clearly cleaves DNA by means of independent reactions at individual sites.

Unlike *Sgf*I, the *Srf*I and *Fse*I endonucleases initially generated some OC DNA during their reactions on their one-site and two-site substrates, pDB7 and pDB8, respectively, and only later gave FLL DNA. But like *Sgf*I, the rates at which *Srf*I and *Fse*I utilized the one-site substrate were the same as those on the two-site substrate, and the two sites in pDB8 were cleaved sequentially at equal rates (data not shown). Thus, both *Srf*I and *Fse*I also cleave DNA through independent reactions at individual sites. Indeed, the initial liberation of OC DNA during the reactions of these two enzymes indicates that they sometimes dissociate from the DNA after cutting one site in one strand.

*Sgr*AI, on the other hand, behaved differently on a one-site substrate, pAT153, compared with a two-site substrate, pDB7 (Fig. 4). In a reaction buffer containing 50 mM KOAc, *Sgr*AI cleaved its single recognition site on pAT153 in two stages: first in one strand to give OC DNA and only later in the second strand to linearize the DNA (Fig. 4*a*). Yet, under the same conditions, *Sgr*AI cleaved pDB7 in a highly concerted manner; only small amounts of the OC and FLL forms were released. Instead, the majority of the SC DNA was converted directly to the products cut at both sites, without a detectable lag phase (Fig. 4*b*). Each turnover of *Sgr*AI on a DNA with one recognition site thus results in the cleavage of one phosphodiester bond, while most of its turnovers on a DNA with two sites

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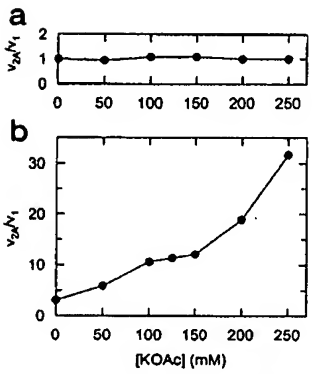

FIG. 5. **Ratio of rates on one- and two-site substrates for *Sgf*I and *Sgr*AI.** Reactions at 37 °C contained 50 units·ml^{-1} restriction endonuclease and 20 nM DNA (with either one or two recognition sites for the enzyme in question) in 33 mM Tris acetate (pH 7.9), 10 mM magnesium acetate, 0.5 mM dithiothreitol, and KOAc at the concentration indicated on the *x* axis. *a*, the endonuclease was *Sgf*I, and the one- and two-site substrates were pDB7 and pDB8, respectively. *b*, the endonuclease was *Sgr*AI, and the one- and two-site substrates were pAT153 and pDB7, respectively. In both cases, initial rates for the utilization of the one-site substrate (v_1) and the two-site substrate (v_{2A}) were measured at each concentration of KOAc, and the ratios (v_{2A}/v_1) are plotted on the *y* axis.

result in the cleavage of four phosphodiester bonds. Furthermore, the rate at which *Sgr*AI utilized the two-site substrate (v_{2A} = 1.52 nM·min^{-1}) was faster than the one-site substrate (v_1 = 0.32 nM·min^{-1}). The different kinetics of *Sgr*AI on substrates with one or two recognition sites therefore eliminate the possibility that this enzyme acts through independent reactions at individual sites. In addition, while the enhanced reaction rate on pDB7 is consistent with *Sgr*AI being a type IIe enzyme, the lack of accumulation of FLL DNA during this reaction discounts this possibility.

The 5-fold difference in the rates of the *Sgr*AI reactions on two- and one-site substrates is, however, considerably smaller than the 20-fold difference recorded with *Sfi*I (21, 22). Hence, the diminished yield of FLL DNA during the *Sgr*AI reaction on pDB7 (Fig. 4*b*) might not be due solely to concerted action at two recognition sites, in the manner of *Sfi*I. Instead, it may be due, at least in part, to processivity along the DNA. The reactions of *Sgr*AI on pAT153 and pDB7 were therefore examined at varied ionic strengths; typical reaction records at an elevated ionic strength are shown in Fig. 4, *c* and *d*. In parallel, the reactions of an orthodox enzyme, *Sgf*I, were also examined at varied ionic strengths. *Sgf*I is optimally active in the presence of NaCl, but *Sgr*AI is largely blocked by NaCl (data not shown), so the comparison between *Sgf*I and *Sgr*AI was made by using KOAc to vary the ionic strength. The rates at which *Sgf*I cleaved its two-site and one-site substrates both declined progressively with increasing concentrations of KOAc (data not shown). Nevertheless, the ratio of *Sgf*I activities on the two substrates, v_{2A}/v_1, remained at unity at all ionic strengths tested (Fig. 5*a*). (Similarly, the v_{2A}/v_1 ratios for both *Srf*I and *Fse*I were unaffected by doubling the ionic strengths of their reaction buffers (data not shown).) The rate at which *Sgr*AI utilized its one-site substrate also declined progressively with increasing KOAc concentrations (Fig. 4, *a* and *c*). In contrast, the rate at which *Sgr*AI utilized its two-site substrate remained essentially constant at KOAc concentrations of ≤200 mM (Fig. 4, *b* and *d*) and was only reduced at ≥250 mM KOAc. Consequently, the ratio of the reaction rates of *Sgr*AI on its two- and one-site substrates increased from 3 to 30 as the KOAc concentration was raised from 0 to 250 mM (Fig. 5*b*). The diminished yield of FLL DNA during the *Sgr*AI reaction on pDB7 at 50 mM KOAc (Fig. 4*b*) therefore cannot be due to processivity.

At all concentrations of KOAc tested, *Sgr*AI initially cleaved its one-site substrate in just one strand of the DNA to give the OC form (Fig. 4, *a* and *c*). However, although the rate of utilization of the two-site substrate for *Sgr*AI remained constant as the KOAc concentration was increased to 200 mM, the initial products from its reactions on pDB7 at high ionic strengths differed from those at low ionic strength. At KOAc concentrations of ≤100 mM, *Sgr*AI cleaved its two-site substrate in a highly concerted manner, converting almost all of the substrate directly to the final products cut in both strands at both sites (as noted above at 50 mM KOAc; Fig. 4*b*). In contrast, at concentrations of KOAc of ≥125 mM, *Sgr*AI cleaved the two-site substrate in a sequential series of separate reactions, giving first the OC form and then the FLL form and only later L1 and L2 (in all cases, as in Fig. 4*d*). Like *Sfi*I (22), both the difference between the reaction rates of *Sgr*AI on one- and two-site substrates and the degree of its concertedness on the two-site substrate vary with the ionic strength of the reaction buffer. The behavior of *Sgr*AI on its one- and two-site substrates matches the expectations for a restriction endonuclease that acts concertedly at two recognition sites.

Other Restriction Enzymes with 8-bp Sites—The MCS in pNEB193 contains single copies of the 8-bp sites for *Asc*I, *Pac*I, *Pme*I, and *Sse*8387I (Fig. 1*b*). A derivative of pNEB193, pAB1, was constructed with two copies of the MCS in inverted orientation (Fig. 1*b*). On pAB1, the distance between the pairs of recognition sites varied from 717 bp for *Asc*I to 803 bp for *Sse*8387I. The SC forms of pNEB193 and pAB1 were used as one- and two-site substrates for these enzymes. An isoschizomer of *Sse*8387I, *Sda*I (Table I), was also examined in the same manner. Both *Sse*8387I and *Sda*I are from *Streptomyces* species, and they also have in common with *Sfi*I a G:C-rich recognition sequence, as does *Asc*I and all four of the enzymes analyzed above (Table I). The restriction sites that are 8 bp long generally possess a marked preponderance (≥75%) of either G:C bp or A:T bp (1). *Pac*I and *Pme*I provide two examples of the latter (Table I).

When assayed on pNEB193 and pAB1, *Sse*8387I, *Sda*I, *Pac*I, and *Pme*I all behaved on the one- and two-site substrates in the same manner as *Sgf*I (see Figs. 2 and 3). All four of these enzymes gave the same rates for the utilization of the one- and the two-site substrates (data not shown). All four cleaved the two-site substrate in sequential stages: first at one site to give FLL DNA and then at the second site to give L1 and L2, with the same rates for the two stages. This behavior was observed in both the standard reaction buffer for the enzyme in question and at elevated ionic strengths. *Sse*8387I, *Sda*I, *Pac*I, and *Pme*I thus all cleave DNA via independent reactions at individual recognition sites in the orthodox manner for type II restriction enzymes.

*Asc*I, however, showed a distinctive pattern of behavior on pAB1 (Fig. 6). Instead of cleaving this two-site substrate in sequential stages, first to FLL DNA and then after a lag phase to L1 and L2, the reaction of *Asc*I on pAB1 at a low ionic strength yielded less of the FLL DNA than expected for a pathway involving two kinetically equal steps. It also yielded the doubly cut products directly from the start of the reaction rather than after a lag phase (Fig. 6*a*). However, at an elevated ionic strength, the reaction profile for *Asc*I on its two-site substrate conformed to the expectations for a two-step sequential pathway, since it now gave rise first to FLL DNA and only later, after a lag phase, the doubly cut products (Fig. 6*b*).

The reaction of *Asc*I on pAB1 at low ionic strength (Fig. 6*a*) is consistent with a value for v_{2B}, the rate for cutting the FLL

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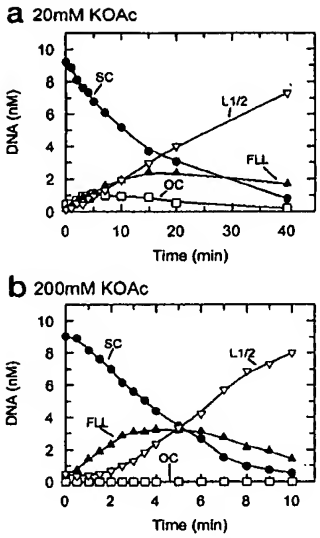

FIG. 6. ***AscI* on a plasmid with two recognition sites.** Reactions at 37 °C contained 24 units·ml^{-1} *Asc*I and 10 nM pAB1 (~90% supercoiled) in 20 mM Tris acetate (pH 7.9), 10 mM magnesium acetate, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, and either 20 mM KOAc (*a*) or 200 mM KOAc (*b*). Samples taken from the reactions at timed intervals were analyzed as above to obtain the concentrations of the following forms of the DNA: SC (●), OC (□), FLL (▲), and total DNA in the two final products cut at both sites (L1/2; ▽).

DNA to L1 and L2, at twice that for v_{2A}, the rate for converting the SC substrate to FLL DNA. A possible reason for this enhancement of v_{2B} over v_{2A} could be that *Asc*I is more active on linear DNA than on SC DNA, but this was discounted. Linear DNA substrates were cleaved by *Asc*I at the same rate as SC substrates (data not shown). Moreover, the data at the elevated ionic strength (Fig. 6*b*) is consistent with a value for v_{2B} that is only 1.2 times higher that that for v_{2A}. In addition, the rates at which *Asc*I utilized its one-site substrate, pNEB193, were the same as those for its two-site substrate, pAB1; at 20 mM KOAc, $v_1 = 0.52$ nM·min^{-1} and $v_{2A} = 0.51$ nM·min^{-1}.

The diminished yield of FLL DNA during the reactions of *Asc*I on its two-site substrate at low strength is therefore not due to concerted action at two recognition sites in the manner of *Sfi*I. Instead, *Asc*I appears to be capable of a high degree of processivity between two recognition sites, even when these are separated by 717 bp. At low ionic strength, *Asc*I presumably binds to one recognition site, cleaves the DNA at that site, and then translocates to the second site by an intramolecular process, without leaving the DNA. One turnover of the enzyme can then result in the cleavage of two separate sites on the DNA. Similar transfers between recognition sites were noted previously with *Eco*RI (12, 13) although over shorter distances than those recorded here with *Asc*I. However, as with *Eco*RI, processivity by *Asc*I is abolished by raising the ionic strength.

Streptomyces Restriction Enzymes—Two enzymes that act concertedly at 8-bp recognition sites are *Sfi*I (23) and *Sgr*AI (this study), which both originate from *Streptomyces* species. To determine whether concerted action at two sites is a common feature of *Streptomyces* restriction enzymes, some endonucleases from *Streptomyces* species that have 6-bp recognition sites were examined by the above procedure. The MCS on pNEB193 has single sites for three such enzymes, *Sac*I, *Sal*I, and *Sph*I (Table I), so pAB1 carries two copies of these recognition sequences (Fig. 1*b*). Previous studies on the kinetics of DNA cleavage by *Sal*I (46) had only used substrates with one recognition site, thus leaving open the possibility that *Sal*I would display enhanced activity on a DNA with two sites. No studies on the kinetics of *Sac*I and *Sph*I have been reported to date. However, *Sal*I, *Sac*I, and *Sph*I all displayed the same activity on the one-site substrate, pNEB193, and the two site-substrate, pAB1, and they all cleaved the latter by means of independent reactions at individual sites (data not shown).

DISCUSSION

Type II restriction enzymes are, conventionally, dimeric proteins that cleave DNA at individual sites (11), but *Sfi*I is a tetramer that cleaves DNA only after binding to two copies of its recognition sequence (25). When the mechanism of *Sfi*I was first characterized (21), no other type II enzyme was known to operate in this manner. *Sfi*I is distinct from the type IIe enzymes such as *Eco*RII and *Nae*I (24). It carries out concurrent DNA cleavage reactions at two identical binding sites for its cognate DNA (23, 27), whereas the type IIe enzymes seem to have two dissimilar binding sites, with the DNA at one site acting solely as an activator for catalysis at the other site (15–18). In this study, a screen was developed to search for other endonucleases that require two sites for their catalytic reactions. The kinetics of a restriction enzyme on plasmids that have either one or two recognition sites for the enzyme were shown to provide a clear cut distinction between the following schemes: separate reactions at individual sites; processivity by translocation from one site to another without leaving the DNA; activation by a second copy of the recognition sequence, as in the type IIe systems; and concerted action at two recognition sites, like *Sfi*I. The test was applied to 12 different endonucleases that recognize 8-bp sequences and/or come from *Streptomyces* species in the belief that these would be the most likely to act like *Sfi*I.

Ten of the enzymes behaved in the conventional manner and cleaved individual sites in independent reactions. These included several *Streptomyces* enzymes that recognize either 8-bp sequences (*Sgf*I, *Srf*I, *Sse*8387I, and *Sda*I) or 6-bp sequences (*Sac*I, *Sal*I, and *Sph*I). The *Streptomyces* enzymes that recognize 8-bp sites all act at G:C-rich sequences (Table I), but conventional behavior was also observed with other enzymes whose 8-bp sites are either G:C-rich, such as *Fse*I, or A:T-rich, such as *Pac*I and *Pme*I. Concerted action at two recognition sites is clearly not a universal feature of the restriction enzymes that recognize 8-bp sequences nor of those from *Streptomyces* species. However, two enzymes deviated from the conventional pattern. In one case, *Asc*I, the cleavage of the two-site substrate matched the expectation for a processive enzyme, at least at low ionic strength (Fig. 6*a*). The translocation of an enzyme from one specific site to another must involve a succession of transient associations with nonspecific DNA. Perhaps *Asc*I dissociates from nonspecific DNA at a slower rate than the other enzymes tested here. The other exception, *Sgr*AI, showed the pattern expected for an enzyme acting concertedly at two recognition sites. None of the 12 enzymes followed the pathway proposed for the type IIe enzymes.

The kinetics of *Sgr*AI on one-site and two-site substrates show that this enzyme needs two sites for optimal activity. On a DNA with one site, *Sgr*AI presumably acts in *trans*, bridging sites on separate molecules, but the resultant complex has too short a lifetime to allow the enzyme to cleave more than one phosphodiester bond before it breaks down (Fig. 4*a*). On a DNA with two sites, *Sgr*AI would act preferentially in *cis*, looping out the DNA between two sites on the same molecule. At low ionic strength, the lifetime of the complex with sites in *cis* is long enough to allow the enzyme to cut both strands at both sites before it falls apart (Fig. 4*b*). The looped complex is likely to have a shorter lifetime at high ionic strength so that the enzyme then has only enough time to cut one phosphodiester bond (Fig. 4*d*). Nevertheless, as expected given the relative

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stabilities of DNA-protein complexes in *cis* over those in *trans* (28), elevated ionic strengths reduced *Sgr*AI activity on the one-site DNA more severely than that on the two-site DNA (Fig. 5). In these respects, *Sgr*AI behaves like *Sfi*I (22). However, while the ability of *Sgr*AI to cleave four phosphodiester bonds in one turnover suggests a tetrameric structure, this has yet to be established. In further experiments on *Sgr*AI,[2] the ratio of its activities on two- and one-site DNA increased with increasing concentrations of the protein, thus raising the possibility that *Sgr*AI may exist as an inactive dimer and that two DNA-bound dimers associate to form an active tetramer.

The recognition sequence for *Sfi*I contains a 5-bp interruption amid 8 specified bp and thus covers 13 bp, longer than is usual for a restriction site (Ref. 33; Table I). Hence, it has been suggested that *Sfi*I is a special case among restriction enzymes and that its unusual reaction mechanism is due to the length of its recognition site (47). However, the recognition site for *Sgr*AI is a continuous sequence of 8 bp. Moreover, it has recently been found that *Cfr*10I, an enzyme that recognizes a continuous sequence of 6 bp, operates in exactly the same way as *Sfi*I (47). Like *Sfi*I (21, 25), *Cfr*10I is a tetramer instead of the dimer proposed previously (48). Again, like *Sfi*I (23, 26), *Cfr*10I interacts with two sites to loop out the intervening DNA (47). The requirement of *Sfi*I for two recognition sites is therefore not a consequence of either the length or the discontinuity of its recognition site but is instead due to a mechanism that now appears to be widespread among type II restriction enzymes. Strikingly, the recognition sequence for *Cfr*10I is the central 6 bp of the 8-bp site for *Sgr*AI. Different restriction enzymes often follow similar reaction mechanisms (*e.g. Eco*RV and *Taq*I) (7, 10). The similarities in mechanism have not, however, been accompanied previously by similarities in recognition sequence.

Endonucleases that need two recognition sites might seem to be less suited for restriction *in vivo* than enzymes acting at a single site, especially with 8-bp sites. In a random sequence containing equal amounts of A, T, G, and C, an 8-bp sequence occurs statistically once per 66 kb (for the degenerate *Sgr*AI site (Table I), once per 16 kb). When 2 megabase pairs of *Streptomyces coelicolor* DNA was analyzed for restriction sites for the *Streptomyces* enzymes that recognize 8-bp sequences (30), the sites for several of the enzymes occurred at frequencies close to the statistical expectation. But interestingly, the enzymes whose 8-bp sites occur most frequently in this DNA are those that need two sites for their reactions, namely *Sgr*AI and *Sfi*I. Recognition sites for *Sgr*AI and *Sfi*I were found in *S. coelicolor* DNA at mean intervals of 1.3 and 2.7 kb, respectively (30). Thus, while *Sgr*AI and *Sfi*I sites may be rare in DNA from other species, they are remarkably common in *Streptomyces* DNA. If the DNA targets for restriction *in vivo* by *Sgr*AI or *Sfi*I are as rich in these sites as *S. coelicolor* DNA, then the enzymes would have no difficulty in locating two sites on the target and restricting it. In addition to these type II enzymes, many other restriction systems employ endonucleases that require two sites on the target DNA: the type III systems (49), some methylation-specific systems (50), and, in some situations, the type I systems (51). The reason why *Sfi*I interacts with two recognition sites is to ensure that it cleaves DNA only at its cognate sequence. It cannot form synaptic complexes with noncognate DNA or with one cognate and one noncognate site, and only the synaptic complex with two cognate sites is catalytically active (27). The same rationale may apply to *Sgr*AI and to *Cfr*10I.

Of the 10 restriction enzymes with 8-bp sites analyzed here and in previous studies (21), only *Sfi*I and *Sgr*AI were found to act concertedly at two copies of their recognition sequences. Hence, it might seem that concerted action at two sites is relatively uncommon among these enzymes, but this may not be so. Almost all restriction enzymes identified to date were discovered by assaying cell-free extracts of bacterial cultures for the fragmentation of a test DNA, usually phage λ or adenoviral DNA (11). However, if an endonuclease needs two copies of an 8-bp sequence separated by an appropriate length of DNA, the test DNA may not be a substrate. For example, there are no *Sfi*I sites on phage λ DNA, and the *Sfi*I endonuclease was discovered by assaying extracts from *S. fimbriatus* on adenoviral DNA (33). Fortuitously, adenoviral DNA contains two *Sfi*I sites separated by 1 kb, which is close enough for a looping reaction by *Sfi*I on linear DNA (22). If the *Sfi*I sites on this DNA had been separated by >10 kb, it is unlikely that its activity would have been detected. Hence, concerted action at two DNA sites may be a common feature of the type II restriction endonucleases present in nature but which have yet to be discovered by *in vitro* assays.

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